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THE COAGULATION OF BLOOD.

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Dissertation

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by

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ON THE COAGULATION OF BLOOD.

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Recent investigators of the phenomenon of blood coagulation have come to rather widely divergent conclusions. Morawitz(1) attempting to harmonize the older views of Schmidt, with the later work of Pekelharing, Hammarsten and others, gives four factors entering into the process. Three of these pre-exist in the circulating blood; the fibrinogen, the thrombogen or proferment, and the salts of calcium. The fourth factor arises at the moment of coagulation, and is produced by the disintegration of the formed elements of the blood--the platelets and probably also the leucocytes. This substance is the thrombokinase, which activates the thrombogen in the presence of calcium salts to form thrombin. The thrombin then attacks the fibrinogen molecule and in the manner of a ferment or catalyzer changes the same to fibrin. The thrombo-kinase may also be derived from the various tissues of the body, and when so extracted serve to activate the thrombin and initiate coagulation. The suggestion of a "kinase" as made by Pawlow in the study of the entero-kinase in the intestines is here applied to explain the action of the "zymoplastic" substances of earlier workers.

On the other hand Nolf(2) impressed by the fact that a clot is soon re-dissolved by an act of autolysis, especially in the case of clots of fibrinogen produced by thrombin, sees in the phenomenon of coagulation as the paramount question, not what makes blood clot, but what makes the clot persist. According to his view coagulation is a normal process in nutrition and assimilation, whereby the fibrinogen of the blood is rendered available as food for the tissues. Coagulation is normally and continually going on in the blood. It consists of a quantitative and mutual precipitation of two colloids, the thrombin and the fibrinogen. The thrombin is formed by the union of the "hepato-thrombine," which has its source in the liver and corresponds in a way to the thrombogen of Morawitz, and "leuco-thrombine,"which is derived from the leucocytes and platelets of the blood. In the circulating blood small amounts of thrombin are formed around the leucocytes in the manner described. This precipitates a small amount of fibrinogen as fibrin, which covers the leucocyte with a thin ultra-microscopic layer of fibrin. This layer of fibrin renders the leucocyte neutral to some extent, in the matter of further production of leucothrombine. This precipitated cuticle of fibrin is now at-

tacked by the real enzyme of the thrombin, the "thrombozyme" and dissolved. This antolytic action is a true fermentative process, and serves primarily purposes of tissue nutrition. In abnormal conditions, such as produce ordinary clots, the leucocytes and platelets are destroyed in great numbers, and the excessive amounts of leucothrombine so formed unite with hepato-thrombine to form the active thrombin that produces the clot. The real question here is, what makes the clot persist. This anti-fibrinolytic substance he believes is the hepato-thrombine, to the inhibiting action of which on the fibrinolysis, the clot owes its persistence. Coagulation is, therefore, according to Nolf, a late phylogenetic adaptation. It is a secondary modification for preventing hemorrhage, of a primitive nutritive and assimilative process.

Loeb,(3) approaching the problem from a study of blood coagulation in the invertebrates, assigns the primary rôle in the process to the coagulins. These coagulins may be extracted from the tissues, and when activated with calcium salts have an effect on the fibrinogen, similar to ordinary thrombin. These coagulins are not merely zymoplastic sub-

stances, nor are they kinases. They do not activate any proferment, but independently attack the fibrinogen molecule. Neither are the coagulins identical with ordinary thrombin. They have a much greater specificity than thrombin, and are more stable.

The fact that tissue extracts properly irrigated will not clot carefully prepared fibrinogen solutions, even when $CaCl_2$ is added, is explained by assuming that the repeated salting out has changed the character of the original fibrinogen.

Mellanby,(4) working with the blood of birds, assigns the main rôle in the coagulation of blood to the "kinases." These kinases, extracted from the tissues activate the proferment and form fibrin. The proferment seems bound in a way to the fibrinogen, so that the amount of proferment varies directly with the amount of fibrinogen present. As source of his kinase he used the macerated testes. Kinase is absent in the muscles. The fibrinogen solutions were made by diluting the clear bird's plasma with 20 times its volume of water, acidulating, centrifuging, and re-dissolving the precipitate

in the original volume of water.

The present investigation was carried on under the direction of Dr. W. H. Howell, to whose stimulating interest and many valuable suggestions, most of any credit which this work may have is largely due.

The first requisite in the investigation of blood coagulation is a trustworthy testing solution. The ideal solution would be the circulating blood, if it could be kept in its normal condition. This is of course impossible, at least with mammalian blood, inasmuch as it begins to clot immediately after taking. Recourse must therefore be had to artificial plasmas. It is believed for reasons to be stated further on that the most satisfactory testing plasma is a solution of fibrinogen, prepared essentially after the manner of Hammersten, with perhaps two modifications.

The method of preparation of the fibrinogen solutions used in this work was as follows:-

On account of the fact that the blood of the cat does not "lake" so readily as that of many other mammals, this animal was used in the majority of experiments. The blood was taken under careful aseptic conditions from the carotid

artery, through a canula and short tube either 'oiled' with some neutral oil, or washed out with a 1% solution of sodium oxalate. A 1% Solution of Sodium oxalate was added in the proportion of one part of oxalate solution to nine parts of blood. The blood was then immediately centrifuged, until a clear supernatant plasma was obtained. Sodium chloride was added to a little more than 50% saturation and the precipitate after centrifuging, and washing in saturated NaCl, was redissolved in a 2% NaCl solution of volume equal to original plasma.

A second and a third precipitation in the same manner produced a final precipitate, which was dissolved in 0.9% NaCl. If such a solution of fibrinogen still remained distinctly opalescent, 1 c.c. of a dilute solution of barium chloride, and 1 c.c. of a dilute solution of sodium phosphate were added. The dilution of the two solutions was approximately such that 1 c.c. of the barium chloride and 1 c.c. of the sodium phosphate neutralized each other. The precipitate of barium phosphate so formed gradually fell to the bottom, and with it were swept out the particles giving the opalescence, leaving finally a clear solution of fibrinogen, not very different from the clear appearance of distilled water.

The solution of fibrinogen was then placed in a collodion tube and dialyzed against a large volume of 0.9% NaCl solution for about 18 hours. This insures a practically complete removal of any oxalate remaining, or of any excess of barium chloride or sodium phosphate. It also insures to the fibrinogen solution a known concentration of sodium chloride.

Such test solutions of fibrinogen do not clot spontaneously, even when kept until putrefactive changes arise. They give no appearance of a clot on the addition of calcium salts, showing that no pro-stage remains in the fluid which can be activated by calcium. No clotting occurs either when Ringer's solution is added, such as occurs frequently in fibrinogen solutions, which have been precipitated only once or twice and which remain strongly opalescent. Probably in such opalescent solutions, some of the fibrinogen has already been affected by thrombin present. Experiments made on this point indicate that it is the alkaline bicarbonate in Ringer's which activates the thrombin and so induces clotting.

That solutions of fibrinogen prepared as here described are however not materially changed from the condition found in the blood is shown by the fact that with serum or thrombin they form a typical clot, which resembles in every essen-

tial the normal blood clot. The promptness of the action, and its similarity in every way to clots of other forms of test solutions prove rather clearly that the fibrinogen in question has retained its characteristic properties, and by its purity and stability is peculiarly fitted to study the real coagulating effects of other factors entering into the process. With the fibrinogen solution, the main constituents of normal plasma were tested as to their coagulating properties. As already pointed out in describing the method of preparing fibrinogen, the inorganic salts of the blood in normal (Ringer) concentrations had no effect, either added singly, or together, or in other possible combinations. Similar negative results were found in the case of solutions of lecithin and cholesterin. Carbon dioxide and oxygen gave negative results. The experiments showed, as has long been known, that the initiating factor of coagulation is not among the simpler chemical substances, which exist preformed in the circulating blood.

THE THROMBIN.

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The observation that serum added to a solution contain-

ing fibrinogen will induce clotting dates back to the work of Buchanan. Later Alexander Schmidt succeeded in getting the active substance in somewhat purer form by treating the serum with alcohol, and extracting the dried residue with water. This substance he designated fibrin ferment, or thrombin.

The thrombin used in these experiments was prepared after Schmidt's method. Blood was drawn from an animal and allowed to clot. As soon as the serum was sufficiently pressed out of the clot, it was diluted with twenty times its volume of alcohol (95%) and allowed to stand for a period ranging from several days to several months. The duration of the action of alcohol seemed to have no very appreciable effect on the quantity or strength of the thrombin extracted. After the proteins had been precipitated by the alcohol they were filtered off, dried, and extracted with water. Into this aqueous extract other things as well as thrombin will enter. Evidently the free salts of the serum will be dissolved. Some of the proteins also go into solution, especially if the aqueous extraction is continued for any considerable length of time. Unfortunately therefore such thrombin solutions are not very pure, and offer serious obstacles to a

critical study of the chemical nature of this substance.

If however the extraction is made of short duration, it is possible to secure a thrombin solution, which is very active in producing coagulation, and which nevertheless gives only the faintest traces of protein. The excess of salts may be removed by dialyzing the thrombin solution in a collodion tube against pure water. Experiments soon showed however that the dissolved proteins interfered in no way with the thrombin action, and some of the strongest thrombin solutions were secured by submitting the dried residue not only to prolonged aqueous extraction, but even to prolonged putrefaction, until the entire protein residue disappeared. Such solutions usually gained in amount of thrombin, as the processes of putrefaction continued, the possible explanation for which will appear in another connection. It is evident that the thrombin extracted from the serum is of the greatest importance in the process of coagulation. It appears in the plasma at the time of coagulation and is the agent which effects the change from the liquid fibrinogen to the solid fibrin. It seemed desirable therefore to determine the conditions under which it acts, its origin, and as far as possible its chemical nature.

1. Rapidity of coagulation with thrombin.

The rapidity of coagulation was found to be inversely proportional to the amount of thrombin added, as indicated in the following experiment:

20 cc.	fibrinogen	+ 40	drops	thrombin--	14 min.
20 "	"	+ 20	"	"	26 "
20 "	"	+ 10	"	"	50 "
20 "	"	+ 5	"	"	90 "

The time required for coagulation was determined by the moment when the glass could be inverted without spilling the contents. Such a rate however holds good only below certain limits. If the amounts of thrombin become excessively large, the rapidity of coagulation does not vary in similar inverse proportion. There is soon reached a maximum amount beyond which the further increase of the amount of thrombin no longer decreases the rapidity of coagulation. The evident reasons for this appear in a study of the quantitative relations of thrombin and fibrinogen.

2. Quantitative relations of thrombin and fibrinogen.

The original statement of Alexander Schmidt that minimal

amounts of his "fibrin-ferment" always produced complete coagulation, was soon questioned. It was found that partial clots could arise, or that a second or even third successive clot might form in the same fibrinogen solution or plasma. Careful quantitative experiments seem however not to have been made. For the experiment on this point 500 cc. of clear plasma of four cats were taken, and from the same 500 cc. of fibrinogen solution prepared. This amount was divided into four equal parts. To part one five drops of a tested thrombin solution were added; to part two, ten drops; to part three, twenty drops, and to part four, forty drops. The glasses of fibrinogen were then allowed to stand untouched for twenty-four hours at room temperature, to allow ample time for coagulation. The fibrin from each glass was then collected on a filter, washed, and placed in a weighing bottle, which together with filter had been previously heated several times at a temperature of 110° C. and reduced to approximately constant weights. The weighing bottles with the several amounts of fibrin were then heated for a number of hours at 110°C., weighed, and reheated until fairly constant weights resulted. Final weighing gave the following results:-

5 drops thrombin - - 0.2046 grams fibrin.
10 " " - - 0.3573 " "
20 " " - - 0.6089 " "
40 " " - - 1.5872 " "

It will be seen from these results, that while they fall short of exact mathematical proportionality, yet they reveal very clearly that there is in the formation of the fibrin a definite and direct quantitative proportion. The bearing of such facts on the supposed fermentative nature of thrombin is treated farther on. It need not be pointed out that such quantitative relationships will appear only, when the amount of thrombin is below the maximum amount required to affect the entire fibrinogen. Adding thrombin beyond this maximum amount can of course be of no moment, there being no further fibrinogen available.

3. Effects of temperature on coagulation with thrombin.

Similar amounts of fibrinogen and thrombin submitted to different temperatures showed that temperatures between 17°C. and 41°C. had practically no varying influence on the time of coagulation. In one experiment on this point the following results were obtained:-

Temperature.	Time of clotting.
1°C.	2 hours.
17°	28 min.
21°	28 "
31°	27 "
41°	29 "

The temperature coefficient of coagulation between the temperatures of 17°C. and 41°C. is practically 1. Whether this is to be interpretated that the process of coagulation is essentially a physical one, or at least is overshadowed by physical moments, is perhaps an open question. It is well to recall however that in the experiments here noted, the thrombin was ready formed. The time element in the coagulation as determined by the coagulometer, on freshly drawn blood is a different matter. In this case there is a definite correspondence between the temperature and the rapidity of the coagulation, as indicated in a few figures taken from Addis.(6)

Temperature.	Coagulation time.
°C.	min. sec.
10.25	21, 30
12.25	16, 30
13.5	14, 32
16.5	10, 10
18.5	7, 34
19.5	6, 2
20.5	5, 22

It is obvious that in these latter experiments, additional factors are concerned, which give rise to the thrombin, either processes of secretion, or disintegration of formed elements, or chemical activation, which drop out, when ready-formed thrombin is used.

PROPERTIES OF TROMBIN.

1. Its stability with reference to heat. In Alexander Schmidt's reports of the fibrin-ferment discovered by him, as given in his book "Zur Blut-lehre," he cites as one proof of the fermentative nature of the substance, that it is completely destroyed by boiling. A foot-note however shows that he had overstated this point, inasmuch as he calls attention to the fact, that "ordinarily one finds that boiling does not completely destroy the ferment." Howell(5) also notes the same fact. In aqueous solutions, fairly free from proteins, the thrombin is not wholly destroyed, and the freer the solution is from proteins, which coagulate on heating, the greater is the amount of thrombin which can bear boiling. Ordinary fresh thrombin solutions after several minutes of boiling, still cause typical clotting. It is therefore not so thermolabile as often asserted, and its reaction to heat

could hardly be cited as proof of its essential similarity to other enzymes.

In dried form it withstands a temperature of 135°C. for a half hour. In regaining the thrombin from such dried extracts, it is necessary to submit it for several days to the dissolving action of water. The necessity for this no doubt lies in the excessively hard baking to which the mass has been exposed, and the difficulty with which subsequent extraction takes place. The extraction may be facilitated by allowing putrefactive changes to arise, which probably in a manner to be described later, liberate the thrombin enclosed. The thrombin is however completely destroyed when charred, or ashed. Extracts under such treatment show no trace of its action. The conclusion is warranted that thrombin is not a simple mineral compound.

Recovery of inactivated thrombin. While aqueous solutions of thrombin withstand in large part, the boiling temperature, solutions of thrombin containing proteins are at first completely inactivated by that temperature. That the thrombin has however not been really destroyed is shown by the fact that its action may be recovered by processes which tend to redissolve the protein. This may be done by submit-

ting the solution of inactivated thrombin to the action of a small amount of sodium hydroxide. Not quite so effective is treatment with a dilute mineral acid for a few minutes. In this way amounts of thrombin may be regained depending upon the maximum temperature reached. Instead of the action of the sodium hydroxide or acid, similar results are obtained by submitting the inactivated thrombin solution to putrefactive changes, which evidently act in the dissociation of the protein and the liberation of the adsorbed thrombin. Such a protein-containing solution of thrombin we have in the serum of the blood. Heating this to 56° or 57°C. is usually given as sufficient for destroying the thrombin present, and insuring a thrombin-free serum. This was found not to be strictly true. Active serum may be heated above 57°C. and its thrombin action regained in part at least. If serum is heated to 65°C. for several minutes, it is found inactive when added to a fibrinogen solution. If however such an inactivated serum is treated with a small amount of sodium hydroxide for a few minutes and then neutralized, it will coagulate fibrinogen, whereas a control of heated serum not so activated remains fluid.

It is an old observation that serum soon loses its

active thrombin on standing, and becomes neutral and ineffective in producing a clot. According to older observers it was supposed to disintegrate and to disappear entirely. When later such inactive serum was activated with sodium hydroxide or mineral acids, and a thrombine activity as much as thirty times the original strength of fresh serum secured, it was explained as a proferment in the blood which had been activated and new thrombin formed. Such was the β proferment of Morawitz, in distinction from the α proferment which is activated by calcium alone. Later the disappearance of the thrombin from serum was explained as due to the formation of a metathrombin, which may be changed back to thrombin by the alkali or acid activation referred to.

The parallelism of action between the gradual disappearance of thrombin from serum on standing and its recovery by activation, and the disappearance of thrombin from serum on heating to 65° and its recovery by a similar activation, strongly points to the conclusion that these two things are essentially the same phenomenon, and that the disappearance of the thrombin is probably due to its combining in some loose way with the proteins of the serum, from which it may in turn be liberated by agencies which tend to dissolve in part at

least the proteins present. That the disappearance of the thrombin is due to such a loose combination with the proteins, and that activation is little more than the breaking apart of this connection, seems probable from the further fact that serum, which has become completely inactive on standing, becomes later, if submitted to putrefactive processes, quite active again. It is not probable that there has been any sort of chemical activation here. The probable results are those of splitting and breaking down the organized proteins. Such an active condition often requires a number of days, due to the remarkable resistance shown by serum to putrefactive changes. Whether this union is of a real chemical nature, or not, is not easily told. It seems however clearly more than mere mechanical adsorption, for neither in the standing serum or the serum inactivated by heating to 56°C. is there any precipitation to make mere mechanical adsorption possible. It is evidently here more nearly allied to a loose but truly chemical combination.

Thrombin solutions containing proteins when heated to points above the coagulation points of these proteins, may no doubt carry down the thrombin in a more mechanically adsorbed way.

Experiments make it certain that thrombin does not unite with all proteins. There is a marked specificity in this respect. If for instance serum be taken and allowed to stand, not only will the thrombin originally in it disappear, but it will cause large additional amounts of thrombin to disappear. In some experiments, serum was able to neutralize its own volume of a thrombine solution whose activity as tested on fibrinogen was greater than that of fresh serum. If however thrombin is added to a solution of egg-albumen and allowed to stand for several days, and its coagulating power tested from time to time on fibrinogen solutions, it is found that practically none of the thrombin has disappeared, whereas the serum so tested has lost its coagulating power, unless it is re-activated, or submitted to slight putrefactive changes, when the thrombin re-appears in part. If serum be heated to 80°C. before the thrombin is added, and it is then allowed to stand, it is found on testing with fibrinogen solutions that the thrombin has not completely disappeared. Clotting is evident, although not so immediate and complete after several days as with fresh thrombin solutions. The explanation is not evident. The partial disappearance may be due to the fact that the heating has not completely destroyed

the combining power of the proteins, or that there exist in blood serum albumens which are not thrown down on boiling. That such albumens do exist is shown by the experiments of Howell on this point.

That the union of the thrombin is with some blood protein is strengthened further by experiments which failed to show that thrombin could be neutralized by other known constituents of the blood. Attention was especially directed to cholesterin, which according to - - - - - - - seems to be the substance in the blood to which the "alexins" are bound. But cholesterin solutions, both aqueous and soapy, showed no effect on thrombin solutions after days of standing.

It has been pointed out that the thrombin and fibrinogen unite in quantitatively proportional amounts in the formation of fibrin, and that coagulation in this respect is the mutual precipitation of these two colloids. But its union seems a relatively loose one here. If less thrombin is added to an amount of fibrinogen than is required to precipitate the entire fibrinogen, a partial clot will form. If this clot be removed, no thrombin remains in the fibrinogen solution, and it will remain fluid, or produce another clot if new thrombin is added. But the fibrin removed yields

relatively large amounts of thrombin, when "digested" according to the method of Gamgee, in a 5% NaCl solution at 40°C. for several hours. In this "solution of the fibrin" the thrombin is liberated anew. The same result may be reached by submitting the fibrin to putrefactive processes. As the fibrin dissolves, tests with fibrinogen solutions indicate a larger and larger amount of thrombin liberated. The question naturally arises whether, if it is possible to regain the thrombin as thrombin from fibrin, it may not be possible by proper treatment to regain the fibrinogen as fibrinogen.

ISOLATION OF THROMBIN.

In approaching the problem of isolating the thrombin, experiments were made to determine the media which dissolve thrombin, and those out of which it is precipitated. It is evidently not dissolved by alcohol. In Schmidt's method for obtaining thrombin, it is the dried precipitate after the removal of the alcohol that yields the thrombin. That practically no thrombin is dissolved in the supernatant alcohol is shown by the fact that when such alcohol is slowly evaporated and the residue extracted with water, no perceptible

traces of thrombin are obtained.

It is equally insoluble in ether, in chloroform and in acetone. Dried serum extracted with these liquids show, when the extract is evaporated and re-extracted with water, no traces of thrombin. Aqueous extracts however of the dried serum after having been submitted to the ether, chloroform or acetone for hours, yield the usual amount of thrombin, showing that none has been dissolved out.

But thrombin is very soluble in aqueous solutions. Dried serum, which had been precipitated by excess of alcohol, when shaken with twice its volume of distilled water for fifteen seconds only, showed a considerable thrombin activity.

Attempts to extract the thrombin with water soon showed that not only the free salts of the dried serum, but some of the protein as well, went into solution, especially so, if the extraction was continued a few hours. The salts are readily removed by dialysis against distilled water and it was hoped the thrombin could be separated from the excess proteins by reprecipitating the proteins with alcohol and extracting anew with water. In this manner it was found that the proteins were thrown out in large part in three reprecipitations, but the thrombin activity was greatly reduc-

ed, in fact practically destroyed. Whether this is due to the fact that the thrombin was thrown out along with the proteins is not certain. It is possible that the toxic action of alcohol is more marked in purer thrombin solutions, while in solutions containing considerable amounts of blood proteins it is protected in some unknown way. The method of reprecipitation gave in all cases finally negative results.

To reduce the solution of proteins to a minimum, the dried serum was extracted with water for a period not exceeding one minute. In this short time, as stated, considerable amounts of thrombin were extracted as shown in tests with fibrinogen solutions. Such solutions of thrombin showed only the faintest traces of protein when treated with the ordinary color tests, and remained clear on boiling. Evaporated, only a small residue remains, of a structure wholly amorphous. No evidence of crystals other than those of the salts normally occurring was found. The amounts of this amorphous residue proved insufficient for purposes of a further hydrolysis or digestion to determine the end-products of their cleavage.

That thrombin is however not a nucleo-proteid, as believed by Pekelharing, is shown by the fact that a thrombin

solution may be acidulated with acetic acid, and the nucleoproteids precipitated. If this precipitate is removed by centrifuging, or filtering, and the filtrate at once neutralized, the thrombin activity has not been materially reduced. The neutralization may not be postponed too long, as acids and alkalies, if allowed to act sufficiently long, are toxic in their effects.

While this experiment shows that we are not dealing with an ordinary nucleoproteid, it is more difficult to arrive at conclusions concerning the protein nature of thrombin. There are some facts which argue against its protein structure. As pointed out by Schmidt and others, it is possible to secure thrombin solutions which are quite active and which give only the faintest traces of protein. As stated in an earlier part of this paper it stands boiling, and so differs at least from the ordinary proteins associated with it in the blood.

More significant however is the resistance of thrombin to putrefactive changes. Bottles of thrombin containing large amounts of dissolved proteins were allowed to stand for months, until the putrefactive changes had run their normal course. Such thrombin solutions lost none of their

activity, in fact gained materially in the initial stages of the process. It should be pointed out however that such solutions even after several months of standing still show very distinct protein reactions, so that it would be entirely wrong to hold that the cleavage due to putrefaction had been complete. It is however to the point that while the splitting is not complete, the proteins present are no longer the complex proteins of the blood, but simpler structures on the road toward final hydrolysis. Through these earlier stages the thrombine suffers no appreciable effect.

On the other hand there are properties of thrombin shared by the proteins. It is not readily diffusible. Dialyzed against pure water, it may, as stated before, be separated from salts present without serious loss. It is however slightly diffusible and allowed to dialyze against a smaller amount of water, it may be shown that distinct traces of thrombin appear on the outside after several hours. Like proteins it seems to be broken down by peptic and tryptic digestion.

Solutions of thrombin submitted to ordinary peptic (acid) or tryptic (neutral) digestion, always lost their coagulating activity. But other possibilities than those of regular

peptic or tryptic digestion exist. In the case of the pepsin-hydrochloric experiment, it may be, that the prolonged action of the acid is toxic. Control experiments seemed to show that this was true. In the case of the tryptic digestion, there was the disturbing action of the trypsin itself upon the fibrinogen of the test solution. Relatively small amounts of trypsin (neutral) in fibrinogen solutions induce active lytic effects, before coagulation is effected. It was attempted to destroy the activity of the trypsin by heating to 65°C., and then by careful activation to restore the thrombin. But all such attempts led to negative results, and it seems possible, if not probable, that thrombin is destroyed by the digestive action of both pepsin and trypsin. While it is impossible therefore to speak definitely as to whether thrombin is a protein or not, it is clear that it is far from being a simple inorganic compound. Some of the reactions of thrombin suggest its possible relationship with histones or protamines. Histones or protamines are derived from cellular elements, corpuscles, sperm cells, etc. There is much evidence that the thrombin is in large part a product derived from the secretion or disintegration of the platelets or leucocytes of the blood. Histones (in salt free solu-

tions) and protamines, do not coagulate on boiling. When in other solutions a precipitate forms it may be redissolved in dilute acids, and on neutralization remains in solution, showing that its action is different from a mere acid-albumen. Histones unite readily in neutral solutions with other proteins, such as egg albumen, serum, casein, forming precipitates, soluble in dilute alkalies and acids. The histones are also easily precipitated out of a neutral solution by small amounts of salts, and this fact would, if applicable to thrombin, explain the difficulty with which pure, thrombin-free fibrinogen solutions are made. In all methods for making fibrinogen, where the initial formation of thrombin is not prevented, there will be found a certain parallelism between the amount of fibrinogen and that of the thrombin, or prothrombin. While the protamines are not destroyed by pepsin, both pepsin and trypsin digest the histones. On standing, some of these simpler proteins suffer a species of denaturation, strongly suggestive of the inactivation of thrombin solutions.

Attempts have been made to test experimentally whether relatively pure thrombin solutions give the characteristic histone and protamine reactions, especially the precipitation

out of aqueous solution by ammonia. The work on this point has not proceeded far enough to warrant any definite conclusions, and the histone-like basic character of the thrombin rests on a few striking analogies only.

SOURCE OF THROMBIN.

Thrombin exists in serum, but what its antecedents are in the circulating blood has been variously interpreted by different investigators. It has been assumed by many that there exists in the circulating blood a proferment or thrombogen or hepato-thrombin, different names by different authors for essentially the same thing. This pro-stage has been referred to the liver as its source by Nolf; by Morawitz it is derived from the formed elements of the blood. Activated by Calcium salts, and by a kinase derived from leucocytes or tissues the pro-stage becomes the active thrombin. By others the existence of a definite thrombogen or proferment in normal blood is denied. A series of experiments were undertaken to determine the existence or non-existence of such a body. It was necessary therefore to treat the blood in such a manner at the very moment of taking, as to prevent the usual changes which occur at that instant and which usher

in coagulation. Especial care was directed to the leucocytes and platelets so that these structures might be prevented from undergoing any marked changes upon the taking of the blood. The procedure consisted in laying bare the carotid artery of the cat, inserting a canula, under best aseptic conditions. The canula and short tube were thoroughly oiled with a neutral sterile oil. On opening the artery, the first few cubic centimetres of blood were rejected. About fifty c.c. of the blood were then led into a large jar of 95% alcohol. Vigorous stirring of the alcohol brought the small stream of submerged blood into general and instantaneous contact with the greatly excessive amount of alcohol and so suffered immediate precipitation, and fixation. The remaining amount of blood drawn from the artery (50 c.c.) was led into a beaker and allowed to clot. The serum from this clot was precipitated with 20 times its volume of alcohol. It was thus possible to have two parts of blood from the same animal, one part precipitated by alcohol, before any coagulative changes occurred, the other part after the process of coagulation had run its course. The two precipitates were then dried, and aqueous extracts made in the usual way for preparing thrombin solutions. Such an extract from the serum showed of

course the usual thrombin activity. Extracts from the blood taken with greatest care under alcohol, showed *no traces* of thrombin. Tested with fibrinogen solutions, no evidence of clotting of any kind appeared. But many similar experiments by Schmidt and others had shown that with greater and greater care in taking blood under alcohol, less and less evidence of "ferment" appeared. It is certain therefore that in circulating blood no appreciable amounts of *ready-formed* thrombin exist. *Does a proferment or thrombogen exist?* To determine this if possible, extracts of the blood were activated with dilute NaOH and dilute mineral acids, without giving any traces of thrombin. Activation with calcium salts in varying proportion proved equally negative. No evidence of any kind could be found that there existed in the blood extract any substance which could be activated by calcium salts to real thrombine activity. It seems therefore improbable that any coagulation caused by the mere addition of calcium salts is due merely to the calcium-activation of a thrombogen.

But as stated above, those holding to the view that a thrombogen exists in circulating blood, assert that the activation is by a kinase in the presence of calcium salts. Extracts

of the alcohol-blood were therefore taken and calcium salts added in proper proportion. They thus lacked only the kinase to produce active thrombin. This kinase according to Morawitz, Nolf, Mellanby and others may come from the tissues. Extracts of tissues which had been carefully irrigated were added, and after standing varying lengths of time, the coagulating power was tested with fibrinogen solutions. In no case was any evidence of thrombin action observed, in spite of the fact that all the supposed elements entering into the active thrombin were present. If it be urged that the treatment with alcohol has destroyed the proferment, it need only be recalled that in the preparation of fibrinogen solutions by the alcohol-precipitation method, the authors assert the existence of the proferment unimpaired. It seems probable from such results that the supposed existence of a thrombogen in circulating blood rests upon very questionable grounds. In fact in reading the literature on this point one is impressed that the reasons for the existence of a thrombogen in circulating blood are drawn more from speculative considerations than from experimental evidence. It would seem that the existence of such a body, certainly tending to complicate the interpretation of the process of coagulation,

should be granted only when definite experimental evidence makes such an assumption necessary. No such necessity exists today, and thrombogen is a theoretical rather than a demonstrable thing. But thrombin does not exist as such in plasma. It must have material antecedents, by which, or from which it is produced. The conclusion seems irresistible that it has its source in greatly accelerated processes which affect the leucocytes or platelets of the blood. Whether the substance formed be by a species of secretory activity or a product of cell disintegration it is impossible to say. But a number of facts point unmistakably to the formed elements of the blood as the source of the pro-stage. Plasma with the corpuscles removed immediately after taking has a greatly increased resistance to coagulation in avian and reptilian blood. In centrifuged blood, experiments showed that parts of the oxalated plasma taken from the region of the tube containing the buffy coat yielded a much more active thrombin solution than a similar amount taken from the top of the tube, where the plasma was, relatively at least, much freer from corpuscles. Substances in actual solution in the plasma could not have shown such an unequal distribution. The absence of thrombin or its pro-stage in the blood taken

under alcohol as just described finds a ready explanation in the assumption that the corpuscles were in this case changed by the action of the alcohol before the normal processes of disintegration were possible. Whether the substance so formed is of an ordinary protein, or histone or protamine character, or a still simpler organic substance is also still largely undetermined. But no matter what its nature may be, it is unable to produce the normal formation of fibrin unless activated by calcium salts. This activation finds its simplest explanation in assuming that the calcium enters chemically into the thrombin molecule. The small amount of thrombin required to precipitate the fibrin, and the small amount of calcium probably entering into the thrombin, makes chemical analyses of this point peculiarly difficult. The variations in amount of calcium due to unavoidable errors, in dealing with such small amounts, may suffice quantitatively for the activation of the thrombin. Such a minimal amount of calcium, Loeb believes enters chemically into the "coagulins." If therefore the free calcium is thrown out of the blood by chemical agents, it remains fluid because the thrombin becomes an active coagulating agent only when combined with this minimal amount of calcium, for the rôle of

which, strontium alone may be substituted with any success.

Sodium-oxalate plasma. The fluidity of oxalated blood is, as has often been pointed out, due to the precipitation of the calcium. It is not due to a mere inhibition exercised by the oxalate. This was shown by taking oxalated plasma and dialyzing the same against 0.9% NaCl for hours. In this way any excess of oxalate is removed, as may be shown by chemical tests. Centrifuging will remove any precipitate, and a fluid plasma is thus obtained which shows no tendency to coagulate, when allowed to stand for days. Additions of very slight amounts of $CaCl_2$ bring about a speedy clotting, showing that the thrombin is lacking only in that salt. A point of interest is in the observed fact that such plasma solutions may stand for several days, and yet give immediate clotting upon addition of $CaCl_2$. The pro-stage of the thrombin, which, of course, is an entirely different thing from thrombogen, and does not pre-exist in blood, does not disappear from the plasma readily. In serum solutions, however, where the activated thrombin is present, this disappears very rapidly as was pointed out in an earlier part of this paper, probably by combining with some specific proteins

in the serum. The thrombin before activation with calcium salts seems not yet to have such combining properties.

The fluoride plasma. The action of fluorides in preventing the coagulation of the blood has been usually held to be due to two things:- the precipitation of the calcium salts and a certain fixation of the leucocytes and platelets preventing the formation of a "kinase." It is probable that neither of these assertions is correct. Fluoride plasma will not clot on addition of $CaCl_2$ even in excess. In this it reacts very differently from the oxalated plasma. This was interpreted by Arthus and others as an index that the fluoride plasma was free from any proferment derived from the leucocytes.

Fluoride plasma remains fluid indefinitely, even, as stated when calcium salts are added. It clots however very readily on the addition of ready-formed thrombin.

If a fluoride plasma is put in a collodion tube and dialyzed against 0.9% NaCl for 12 hours, the plasma clots, and the serum expressed from the clot is peculiarly rich in active thrombin.

Evidently all the factors to make active thrombin were

present in the fluoride plasma, but were prevented from interacting in the usual way, by that medium. Removal of the fluoride by dialysis, or by extreme dilution, permitted once more this normal interaction to form the active thrombin. The calcium is therefore not precipitated out of solution as with oxalates. It is rather in some way bound to the fluoride. That the actions of oxalates and fluorides on $CaCl_2$ solutions are essentially different is evident in observing the relative amounts of precipitate produced by these two reagents. In the case of the oxalate, the precipitate is massive and distinct and soon falls to the bottom. In the case of fluoride, added in similar concentration, there is little more than a cloudiness, no copious precipitate appears. It is therefore probably not true that the calcium is precipitated. Nor is the second assertion correct, that the formed elements of the blood are "fixated" by the fluoride and so prevented from giving off any pro-stage of the thrombin. The pro-stage is produced in usual amounts. No active thrombin results because the calcium salts are in some loose way bound to the fluoride. On removal by dialysis the loose combination is broken up, the calcium activates the thrombin, and the plasma clots in a perfectly normal

manner. That the fluoride binds not the ready-formed thrombin, but probably the calcium required to activate the pro-stage of the thrombin, is evident from the fact that ready-formed thrombin added to a fluoride solution loses none of its coagulating activity. If the calcium be granted as having entered chemically into the thrombin complex, it means that when so combined it can not be removed by fluorides. The same applies also to oxalates. The calcium of formed thrombin is no longer "free" calcium, but is now organically bound to the complex thrombin molecule. In using fluoride plasma for testing purposes it should be borne in mind that all the factors of thrombin exist in the medium. Coagulation is prevented merely by the fact that the fluoride inhibits the interaction of these factors to form thrombin. Clotting of fluoride blood may therefore readily be an effect of removing this inhibition, and not an evidence of a newly added thrombin-like agent.

DO THE TISSUES YIELD A COAGULATING AGENT?

One of the most involved chapters in the study of blood coagulation has been the question, whether there are derived from the tissues as extracts or otherwise substances which

either clot the blood directly as thrombin does, or which exert a favorable and accelerating influence in the process. Schmidt's "zymoplastic substances" were supposed by this investigator to greatly facilitate, if not to make possible, the ordinary process of clotting. The work of Hammersten made clear however that coagulation was a property belonging to the fibrinogen alone, and that Schmidt's zymoplastic substances were not necessary to produce a normal clot. Perhaps this induced investigators who saw a distinctly favorable influence exerted by the extracts of tissues, to interpret this effect not from the side of fibrinogen, but rather from the side of the thrombin, and the effects were ascribed to tissue-thrombins. This is the view of Nolf, who holds that in addition to the leuco-thrombine of the blood, the tissues produce histo-thrombins. The extract from the walls of blood-vessels he designates as vaso-thrombine. These views are essentially the same as those of Morawitz who designates the active substance in the tissue extracts as Thrombo-kinase. They do not directly induce clotting, but serve to activate the proferment in the blood. This is further the view of Mellanby, who in his experiments on coagulation, used the macerated testes of the cockerel as his source of "kinase."

This "kinase" is held to activate the proferment of the blood and so produce the active thrombin which clots the fibrinogen. If this view differs at all from the views of Morawitz it is perhaps in the emphasis it lays on the kinase. The active thrombin stage is a short intercalation in the events of coagulation. The essential action is a "kinase" action.

On the other hand, Loeb, approaching the question from the study of coagulation in invertebrate blood, sees in the extracts of tissues, not kinases, but definite "coagulins" which are able to attack the fibrinogen molecule directly and form fibrin. These coagulins are not identical with thrombin, but have several well-marked characteristics, especially that of their specificity. The question as to the existence of such "kinases" or "coagulins", or "zymoplastic substances" was submitted to careful study in the following experiments:-

Fibrinogen solutions were made from the blood of an animal, in the manner already described. The organ or tissue from the same animal, to be studied for the presence of active coagulating substances, was then irrigated by insertion of a canula in its main artery and the ligaturing of neighboring arteries. The fluid used was Ringer's solution with

which the irrigation was continued for several hours. In this way the organ or tissue was thoroughly freed from its contained blood. The proportion of salts was but slightly disturbed, and the tissue-cells were not seriously modified. That such is the case is fairly evident from the fact that the mammalian heart, so irrigated, beats on for hours. There is therefore no reason to believe that the careful and prolonged irrigation has materially changed the tissues. Muscles, for instance, that still contract have also probably retained the power to produce a kinase or coagulin if such exist. This is assuming that any such "thrombic" substance ready-formed in the cells of the tissue is washed out by the irrigation. But this is not probable, for experiments have shown that thrombin, and so probably similarly acting substances, are only slightly diffusible. After such prolonged irrigation, to remove every trace of blood or serum, bits of the organ or tissue were macerated in 0.9% NaCl and portions of this ground tissue put in fibrinogen solutions. Of another part of the irrigated tissue, filtered saline extracts were made and these tested from time to time on fibrinogen solutions. In not a single case in the repeated series of ex-

periments was there found any evidence of coagulation. All such fibrinogen solutions remained fluid like the controls beside them, while the addition of thrombin induced the ordinary clotting at once. The sweeping statement just made needs a slight modification. In the case of two tissues, the results were not completely negative. One of these was the macerated red marrow of bone. This produced a slight coagulum over its surface, which did not however extend very far into the solution, and there never formed anything approaching a typical clot. Whether this is to be interpreted that there exists in red bone marrow a real coagulating agent is open to question. The marrow remained perfectly red after the irrigation, and extracts of it made were slightly discolored by the hemoglobin. It is possible, remembering the finer structure of this tissue, that perfect irrigation under the circumstances was impossible, and that the tissue still contained blood or serum. The other tissue was the spleen. Macerated and extracted, it produced in fibrinogen solutions small flocculi, which soon settled to the bottom as a white precipitate. There was no evidence of clotting. With these two qualifying statements, the experiments in every case gave not the slightest evidence of the presence of any substance induc-

ing coagulation. There were then evidently no thrombins or coagulins present. Were "kinases" present? These would require prothrombin and calcium salts to produce an active thrombin. The prothrombin being supposed to exist in circulating blood it ought to be extracted when blood taken under alcohol and dried, is subjected to the dissolving action of water. It has been pointed out that the supposed prothrombin in Mellanby's fibrinogen solutions has been procured in essentially the same way. Such extracts treated with calcium and extracts of tissues thoroughly irrigated gave no evidence whatever of the presence of a coagulating agent. Evidently in the chain of argument as usually presented there is a missing link. Either the prothrombin or the kinase or perhaps both are non-existent. Thus throughout these experiments, no hint of a coagulin or a kinase could be discovered. They left unexplained however the obvious facts that some forms of blood or plasma do react immediately and definitely to tissue extracts. The experiments on the blood of terrapins and birds noted by previous observers were repeated. Terrapin's blood taken carefully from an oiled canula, and put in a perfectly clean beaker will remain fluid for days. The blood may be centrifuged and the clear supernatant plasma

is equally or more resistant. If however tissue extracts or pieces of tissue be added, the coagulation is pronounced and immediate. The most apparent explanation is that of a direct coagulating agent present in the extract. But this blood or plasma can be made to clot equally well and equally rapidly in ways which preclude the presence of such a definite agent. If,for instance,dust particles, loose sweepings, or linty shreds be added, the coagulation is equally prompt, and in a number of experiments was more rapid than with tissue extract. Boiling the tissue extracts was found not to have decreased the coagulating action in the least. Tissue extracts of terrapin were dialyzed against distilled water for twenty-four hours. In this way not only were the free salts removed, but much of the dissolved protein (globulin) was precipitated and the solution seemed relatively much clearer. Such solutions showed a very much diminished effect in producing clotting. It is therefore hard to escape the conclusion that simple physical moments are at play here in facilitating the coagulation of the terrapin's blood.

Similar experiments were made on birds. The blood was taken,under septic precautions, from the femoral artery of a chicken. Such blood, as pointed out by Delezenne, remains

fluid for a long time. Centrifuged, it gives a clear plasma which will stand (under cool conditions) for days. If to such blood tissue extracts of the bird or terrapin either, be added, clotting as in the case of terrapin's blood is immediate, and the clot is firm. If the clear plasma is taken, the clotting is not quite so rapid. It is held that these tissue extracts contain a "kinase" which activates the prothrombin to thrombin. But the bird's blood or plasma clots with practically the same rapidity and firmness if dust particles are generously added. Bits of down-feathers introduced bring about a speedy clotting. Surely there can be no question of a "kinase" in these instances. Ringer's solution hastens the coagulation time. Macerated testes when dialyzed against distilled water for twenty-four hours and then filtered has lost much of its accelerating property. Whether this loss is due to the absence of the mineral salts in the extract, or the precipitation and removal of the cellular debris from the same, is not clear. Probably both factors enter into the result. Boiling the testes extract, or other tissue extracts, had no effect, showing that the accelerating cause is not in the least thermolabile, and so differs very greatly from thrombin, which boiled in protein solutions suffers a marked diminution

in strength. Mellanby states that he could find no kinase in muscular tissue. In these experiments muscle extract was practically equally effective as compared with extract of testes. In Mellanby's experiments the fibrinogen had been prepared by dilution of plasma with water, acidulation, the centrifuging of the precipitate, and its final re-solution "in toto" in water. Such solutions clotted slowly with calcium salts alone, and very rapidly with extract of testes. Extract of muscle however no longer sufficed. It is difficult to escape the conclusion that the fibrinogen solution was but slightly more purified than the blood itself, and showed essentially the same reactions. Remembering the ease with which bird's blood clots on the addition of a great number of entirely foreign substances, with a rapidity and firmness not materially below that exercised by these tissue extracts, further investigation would seem necessary before that effect with tissue extracts could be attributed to a specific "kinase". The supposed absence of such a "kinase" in muscular tissue, where it would appear to be of the greatest significance and value, if "kinases" play the important rôle assigned to them, is not easily understood. It has been suggested that the resistance of the blood of bird and reptile to coagulation may be due to

the relatively greater difficulty with which the formed elements disintegrate, and that the explanation of the action of extracts especially those containing cellular detritus, and of dust-particles, lies in the fact that these facilitate the breaking down of the corpuscles. On the other hand it may be that the foreign substances added from "nuclear" points of coagulation, and so exercise a physical influence in the main. That the tissue extracts of bird or terrapin do not contain an active coagulating agent at all similar to thrombin is shown by the following experiment: Extracts of carefully irrigated tissues of bird or terrapin when added to fibrinogen solutions show no coagulating property whatever. The fresh serum of bird or terrapin causes a prompt clotting showing that the thrombin is present here in the usual manner. This also calls attention to the fact that tissues not thoroughly irrigated will of course show clotting properties, in direct proportion to the amount of blood remaining, and that extracts made from such un-irrigated tissues must give untrustworthy results.

ANTI-THROMBINS.

The question whether anti-thrombins exist in the blood has been variously answered by different observers. In this series of experiments only one clear evidence of anti-thrombic action was met. This was the neutralizing effect of serum. Fresh serum soon becomes inactive, and relatively large quantities of a strong thrombin solution disappear entirely in a short time. These may be recovered in part in least by proper alkali or acid activation. There is no reason why a similar neutralizing action might not be exercised by the circulating plasma, and in this manner the small amounts of thrombin which probably are being formed all the time are destroyed. Direct experimental evidence on this point is lacking. The argument of Morawitz, for the existence of an anti-thrombine in blood, because very small amounts of thrombin produce only partial clots, which do not proceed farther, in oxalate or fluoride blood, is to be differently interpreted. This partial clotting is due to the quantitative relations which hold between thrombin and fibrinogen.

Bordet and Gengou, following the general methods of preparing immune sera, injected rabbit serum into the guinea-pig, and so produced a serum which retarded, or destroyed the

coagulating action of rabbit serum. This anti-action was upon the thrombin and not upon the fibrinogen. They found that when fresh rabbit serum which had been heated to 58°C. was added to the fresh serum of the guinea-pig and this mixture added to the bird's plasma, it clotted, even though the active ferment of the fresh rabbit serum was supposed to have been destroyed by the heating. If however the serum of a rabbit, previously injected as described, was heated to 58°C. and then added to the serum of the guinea-pig, and this mixture added to bird's plasma, there was no clotting. Accordingly it was interpreted that there existed in this immune serum a specific anti-body, not destroyed by a temperature of 58°C., which had inhibited the action of the thrombin in the guinea-pig serum.

The immune serum when not heated will however at once clot bird's plasma. The anti-body is therefore not specific for its own serum. Whether we are dealing here with the formation of a real anti-body, or whether the results are more involved, and due to the play of other factors, further investigation must show. As Morawitz states, the experiments are not fully conclusive, and admit of different interpretation. Whether specific anti-thrombins could be produced artificially

was a question which the Dorpat investigators very early made a subject of experimental inquiry. They injected "fibrin ferment" intra-vascularly, and noted the possibility of forming thrombi or when these did not occur, of forming anti-bodies. The results were generally negative. With the more recent production of various specific anti-bodies, such for instance as the anti-chymosin of Morgenroth, it seemed desirable to repeat the work on the possible production of an anti-thrombin, which should be specific for the same blood. The presence of such an anti-thrombin in excess would at once be indicated by a condition of haemophilia. In the first experiment a medium-sized dog was taken, and the time of coagulation of its blood determined, by taking a small amount from the vein of the ear, and following the usual clinical method. Injections of a sterile thrombin solution were then made every other day, in doses beginning with 2 cc. and increased 2 cc. each succeeding time. The sterile thrombin solution was made by extracting heated dried serum with distilled water. To insure an active solution, the thrombin was tested on fibrinogen. After such injections covering a period of two weeks, the animal was killed. The blood clotted in the normal time, and the serum, when tried on fibrinogen solutions

was found to have the usual coagulating strength. There was therefore no evidence, of any kind, of the existence of an anti-thrombin. Nor was there any evidence of intra-vascular thrombi. The disappearance of the large amounts of thrombin injected is not easily explained.

In the second experiment a rabbit was taken, as this animal reacts more promptly to such treatment. Sterile active thrombin was injected sub-cutaneously in amounts beginning with 2 c.c. and gradually increased to 20 c.c. The coagulation time of the blood was determined from time to time. It was found that the injection of the thrombin was entirely without effect. In neither case was there any evidence of haemophilia and the serum after clotting contained the usual amount of thrombin. These results seem to indicate that the blood does not produce a specific anti-body for thrombin, or if it does, the anti-body is not produced in the great excess usual with anti-bodies. Only so much is produced as is necessary to neutralize the free thrombin present. It has been pointed out however in an earlier part of this paper that fresh serum is able to neutralize not only its own active thrombin but added excessive amounts. The neutralization was believed to be due to a loose combination with some specific

albumen of the blood. It is possible that the disappearance of thrombin from the circulating blood may be due to the same action. The observed fact that anti-bodies can not be artificially produced by the injection of relatively simple organic substances, but are limited more specifically to substances essentially protein, may mean that the thrombin is of the former class, although this observation unsupported by other evidence would be inconclusive as to its non-protein structure.

General Conclusions.

The experiments here reported point to the following general conclusions:

1. The coagulation of blood is probably not a fermentative process. Definite statement on this point is complicated by the present unsatisfactory knowledge of the exact nature of ferments and their action. But the coagulation of blood is clearly a quantitative reaction, and a mutual precipitation of two colloids, fibrinogen and thrombin. The partial clots which never proceed farther, the successive clots which may be formed by successive additions of definite amounts of

thrombin, and the approximate equality in amount between the sum of these successive clots and a maximum initial clot, indicate a definite quantitative proportionality. Careful quantitative weighings of the fibrin formed, as reported in the earlier part of the paper, showed such a direct proportionality between fibrinogen and thrombin, as would be required in a definite chemical combination of two substances. The further resistance of the thrombin in aqueous solution relatively free from dissolved proteins to boiling temperatures is a property not usual with the ordinary catalytic ferments. The recovery of thrombin from protein solutions, such as serum, which have become inactive on standing, or by being heated to 60°C. points toward the same conclusion. Such a recovery whether by activation with alkali or acid, or by putrefactive changes, is believed to depend upon the dissociation of the combination of thrombin with some specific albumen other than fibrinogen. In a similar way the partial recovery of thrombin from fibrin, according to Gamgee's method, is more than the solution of merely adsorbed amounts. The three main reasons given by Schmidt originally as proofs of the truly fermentative nature of his ferment are as follows: (1) Minimal amounts of fibrin-ferment will produce a complete

coagulation.

(2) The artificial serum contains after coagulation unused ferment with which several successive clots may be produced.

(3) The ferment is destroyed at 100°C.

The first point has been shown to be incorrect. Minimal amounts produce correspondingly small clots. The quantitative proportionality holds with all sub-maximal amounts of thrombin added to a fibrinogen solution. Nor is it possible to find unused thrombin in a fibrinogen solution if a sub-maximal amount has been added. If the clots are removed after 4-24 hours, the remaining fibrinogen solution remains fluid, but clots promptly again on addition of further amounts of thrombin. The probabilities are therefore that the action of the thrombin is in no way that of an ordinary catalytic ferment, but that the formation of the fibrinogen is essentially the mutual precipitation of two colloids from the plasma.

2. It has been impossible to find experimental evidence for the assumption that there exists preformed in the blood a thrombogen or pro-ferment which is activated by a second agent in the presence of calcium salts.

3. The existence of "kinase" or coagulins in the various

tissues is improbable. With carefully prepared fibrinogen solutions, extracts of tissues irrigated to remove every trace of thrombin-containing blood, no evidence of clotting occurs. When the addition of such extracts produces coagulation in bloods of bird, or reptile, similar results can be secured by the addition of substances, which preclude the presence of definite coagulating agents. Such results render very probable the assumption that in such plasmas all the factors of coagulation are potentially present and the addition of tissue extract or other foreign substance is a secondary action altogether.

4. The active coagulating agent is thrombin, whose properties and reactions were the chief questions of inquiry in this work. It is derived from the formed elements, in all probability, at the moment of their disintegration. In this pro-stage it is inactive in the blood. To become an active precipitating agent it is necessary that minimal amounts of calcium (strontium) salts enter chemically into the molecule complex. Such calcium salts exist in the normal plasma, and the action of sodium oxalate depends on the precipitation of the calcium out of the solution. The action of sodium fluoride consists in preventing the combination of the calcium

with the pro-stage of the thrombin, the calcium being bound but not actually precipitated. Removal of the fluoride therefore by dialysis or dilution permits the inhibited combination, and active thrombin results.

5. A specific anti-thrombin can not be produced artificially by injection of active thrombin, but the serum contains specific albumens which neutralize the thrombin even in excessive amounts, and so perhaps figure in maintaining the fluidity of the circulating blood.

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BIOGRAPHICAL.

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Louis John Rettger was born in Huntingburg, Indiana, October 19, 1867. He received his primary education in the public schools of the State of Indiana. In September 1884, he entered the Indiana State Normal School, graduating from the same in June 1886. He entered the Johns Hopkins University in October 1886 and received the degree of Bachelor of Arts in June 1888. He was a graduate student in the Johns Hopkins University 1888-1889, serving as Laboratory Assistant in Biology. He was appointed instructor in Biology at Indiana University, September 1890, and received the degree of M.A. from that Institution in June 1891. September 1891,he was appointed Professor of Physiology at the Indiana State Normal School,which position he still holds.During the year 1896-97, he attended the Universities of Heidelberg and Berlin, one semester each. In October 1908, he re-entered the Johns Hopkins University, pursuing studies in Physiology, Botany and Zoology.

Johns Hopkins University,

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